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Regularities of Ultrasonography of Suspensions of Alumina Nanoparticles in Biological Media

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Abstract—This paper studies the echo-contrast properties of an alumina nanopowder suspension using ultrasonography (US) fully corresponding in its characteristics to the techniques of medical ultrasound diagnostics of organs and tissues. The purpose of this study was to search for the possible effect of the ionic and protein composition of the biological medium on the intensity of the reflected echo signal of the contrast material based on nanoparticles. It was found that the pH of the blood promotes the maximum use of echo contrast options of alumina nanopowder suspensions. Particle size measurements in the suspension using the dynamic light scattering technique showed the stabilizing effect of blood serum and plasma on the nanopowder suspension, resulting in the attenuation of the echo signal. The data offer a basis for the development of new contrast materials based on nanoparticles for the ultrasound imaging of the heart and blood vessels. The considered mechanisms of the established phenomena make it possible to elucidate the processes of interaction of metal oxide nanoparticles with biological molecules.

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INTRODUCTION

The current state of medical diagnostics, particularly cardiodiagnostics, is based on an assumption that the most accurate and immediate information on pathological changes in the cardiovascular system can be obtained through the assessment of structural features and functions of myocardium and blood vessels, including coronary arteries of the cardiac wall. Accordingly, complex diagnostic techniques for heart visualization on the basis of X-ray, magnetic resonance, radionuclide, and ultrasonic tomography have become available to physicians in recent years.

The high efficiency of diagnostic techniques is achieved through the application of contrast substances, which make it possible to elaborate the structural features of the heart and vessels. Contrast agents for cardiodiagnostics gained wide recognition primarily in the application of the X-ray and magnetic resonance examination of patients. However, despite the low price and ready availability of ultrasonography (US), a contrast of the heart and vessels did not gain proper acceptance due to defects of the corresponding materials.

Free or encapsulated gas microbubbles are generally used as contrast agents for US diagnostics [1, 2]. Such materials are soon destroyed in the bloodstream under exposure to ultrasound, which may be accompanied by lesions of the vessel and cardiac tissue under microbubble cavitation [3]. Therefore, the application of contrast agents based on suspensions of solids, e.g., collagen particles, appears to be preferable [4, 5].

Our early study considered the fundamental possibility of applying aqueous suspensions of metal oxide nanoparticles as contrast agents for ultrasonic diagnostics [6]. In particular, dependences of the reflected echo-signal intensity on the metal oxide type and regularities of the structure of the powder and the formed aggregate nanoparticles in the aqueous medium were established. It is shown that alumina is characterized by the highest echogenicity in the series of metal oxides of Al, Fe, and Zr. These data served as a basis for the development of a new technique of applying nanomaterials for heart and vessel US [7].

This work is dedicated to studies of the properties of the echo contrast of the alumina nanopowder suspension under its interaction with biological media. The purpose of this study was to search for the possible effect of the ionic and protein solution composition on the intensity of the reflected echo signal of the contrast material. The regularities of US of the alumina suspension under the variation of pH of the medium and the serum and blood plasma concentration are shown, and the possible mechanisms of the observed phenomena are considered.

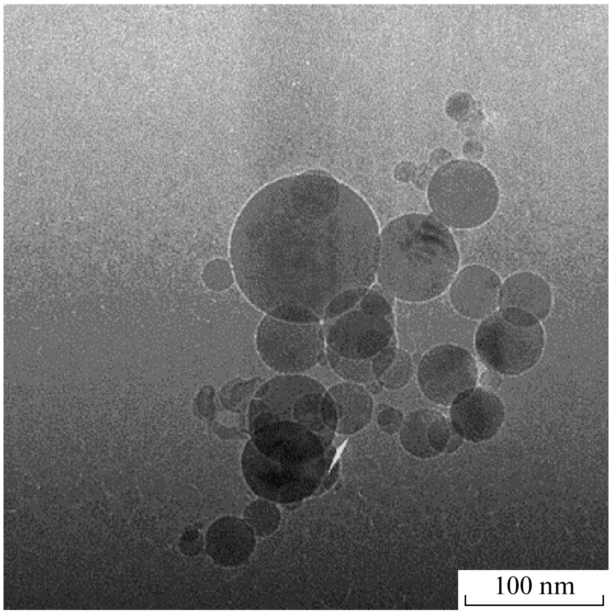

Fig. 1. Electron microphotographs of particles of Al_2O_3–95n nanopowders. A JEOL JEM 2100 microscope.

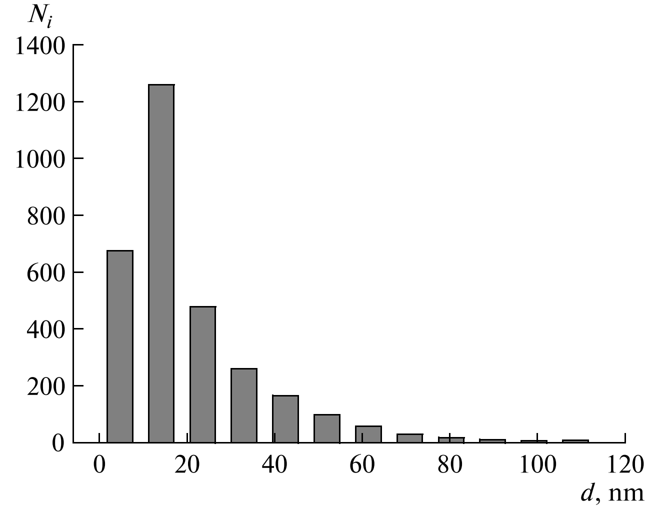

Fig. 2. Size distribution histogram of alumina nanopowder particles.

MATERIALS AND METHODS

Alumina nanopowder obtained using the electric wire explosion (EWE) technique was used in the work [8]. The powder is characterized by a spherical particle shape with a relatively smooth surface (Fig. 1). The numeric particle size distribution of alumina nanopowder is rather broad (Fig. 2). It can be described well using a lognormal distribution function with a mode of 16 nm and dispersion of 0.66. The specific surface area of the nanopowder measured using the low-temperature nitrogen sorption (BET) technique on a Micromeritics TriStar3000 analyzer was 44 m^2/g. The average particle diameter calculated on the basis of the specific surface area for spherical particles was 37 nm, which agrees well with the value of the diameter averaged over the surface of all particles (43 nm) using a lognormal distribution with the above parameters.

The Al_2O_3 nanopowder was dispersed in the physiological solution (0.9% NaCl) using a Cole-Palmer CPX 750 ultrasonic processor. Ultrasonic treatment with a power of 300 W was carried out in cycles (10 s of exposure and a 10-s pause) for 10 min. A 5% basic suspension was prepared. Its pH was adjusted by the addition of 0.1 N HCl and NaOH solutions.

The biological media used were human plasma (Control plasma P, Dade Benring) and horse plasma (Biolot, St. Petersburg). The basic suspension was diluted by a serum using different ratios; as a result a series of suspensions with a serum content of 20, 33, 50, 66, 75, and 85% was obtained. The nanoparticle content in these suspensions was 4.0, 3.3, 2.5, 1.6, 1.2, and 0.7%, accordingly. A series of suspensions with the same plasma ratios was prepared in a similar way. Suspensions of similar concentrations based on the physiological solution (0.9% NaCl) were used as measurement controls.

The values of the hydrodynamic particle diameter in suspensions and their electrokinetic potential were obtained using the techniques of dynamic and electrophoretic light scattering using a Brookhaven ZetaPlus universal analyzer.

The echo contrast properties of suspensions were estimated in 2D mode using a Siemens Sonoline Adara US setup with a SIEMENS 7.5L45s Prima/Adara linear sensor. The dynamic ultrasonic exposure range was 36 dB; the frequency was 5 MHz. The studies were carried out on a hydrodynamic setup modeling the blood channels [6]. The linear flow rate was 3×10^{-2} m/s, and the diameter of a model silicon tube vessel was 4.5 mm.

The reflected signal intensity was determined on the basis of the image brightness. This is related to the fact that the so called B mode is used in modern sonicators for visualization where the screen glow brightness in each scan plane point is proportional to the amplitude of US vibrations reflected from heterogeneities. The device settings (amplification and maximum brightness) were constant in all experiments to provide similar visualization conditions. The video image was processed using a measurement of the average brightness of image details (pixels), $(I_i - I_0)$, where I_i is the image region with a size of 5×5 pixels for the nanoparticle suspension and I_0 is the background brightness (the brightness of the aqueous medium image outside the tube modeling the blood vessel). The average image brightness was given as a percentage vs. the maximum permissible value of the grayscale image brightness being 255 for all modern US devices.

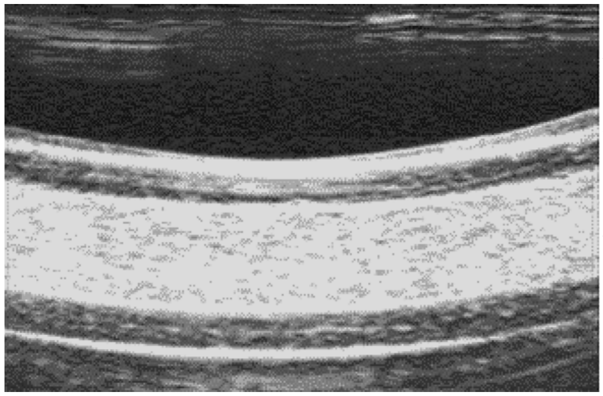

Fig. 3. Ultrasonic image of a tube with the basic Al_2O_3 suspension.

RESULTS

Figure 3 shows the longitudinal section of an ultrasonic image of the suspension flow in a model vessel. One can clearly see the silicon tube wall against the black background (water) and a suspension of nanoparticles under it with an average image brightness of 75.8%.

The pH of the basic suspension was 8.15. Figure 4 shows the dependence of the brightness of the ultrasonic image on the pH of the Al_2O_3 nanoparticle suspension. One can see that the relationship features a maximum in the pH range of 6.3–7.4, where the image brightness reaches 82%. Therefore, the acoustic resistance of the nanoparticle suspension in the pH range corresponding to the normal acid–alkali balance in blood (7.25–7.45) is maximal. In the course of acidification or alkalization of the medium, the echo contrast ability of the suspension decreases drastically. The image brightness decreased from 25 to 35% in the extreme experimental points at pH = 2.57 and pH = 10.4, accordingly.

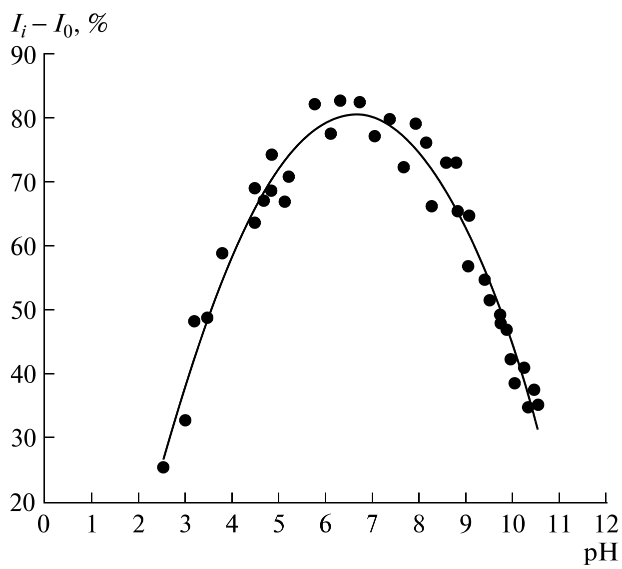

Fig. 4. Dependence of the brightness of nanosuspension images on the value of pH of the physiological solution. *x* axis: pH values; *y* axis: average image brightness.

Figure 5 compares the weight–size distributions of nanoparticles in (*1*) the initial powder, (*2*) an aqueous suspension, and (*3*) a suspension in the physiological solution. One can see that Al_2O_3 nanoparticles are aggregated in aqueous media. Thus, the hydrodynamic diameter of aggregates in an aqueous suspension is about 150 nm, while the weighted average of the particle diameter calculated on the basis of the lognormal distribution of the initial nanopowder is 57 nm. This ratio is typical for alumina nanopowders obtained using the EWE technique, because the primary aggregates present in the air–dry powder are largely preserved in sedimentation–stable aqueous suspensions [9]. A transition from the aqueous medium to the physiological solution medium results in the secondary aggregation of Al_2O_3 nanoparticles: the average

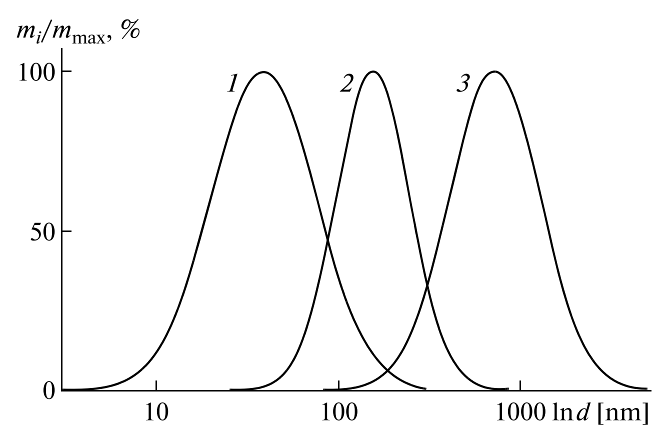

Fig. 5. Weighted particle size distribution in (*1*) the initial nanopowder, (*2*) aggregates present in the aqueous suspension, and (*3*) a suspension based on the physiological solution. *y* axis: weight percentage; *x* axis: overall volume of the most frequent aggregates (particles).

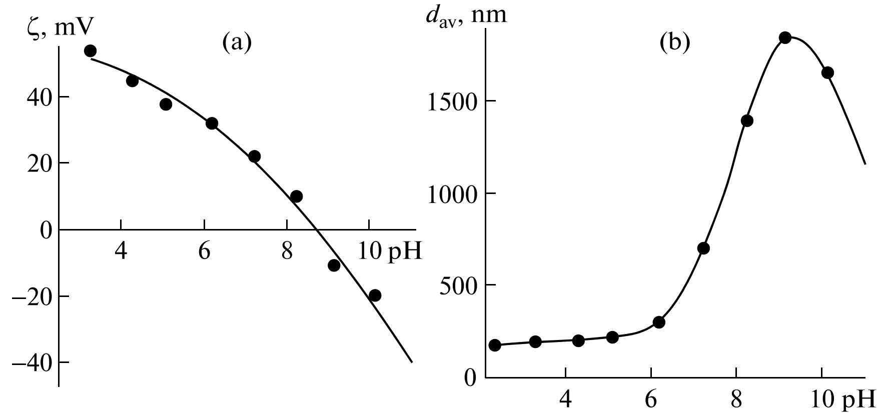

Fig. 6. Dependence of (a) the ζ potential and (b) the average aggregate diameter in the Al_2O_3 suspension on pH.

hydrodynamic diameter of aggregates grows considerably and approaches 1 μm.

Figure 6 shows the effect of pH on secondary aggregation and the ζ potential in Al_2O_3 suspensions in the physiological solution. One can see that the ζ potential assumes high positive values exceeding +30 mV in the range of pH < 6. Under these conditions, the suspension is stable and the average hydrodynamic diameter of aggregates does not exceed 200 nm. However, when pH approaches the physiological standard, the ζ potential decreases (Fig. 6a), which results in the destabilization of the suspension and an increase in the aggregate diameter (Fig. 6b). The further increase in pH and transition to the alkaline media results in a drastic increase in the diameter of aggregates and coagulation of the suspension. The dependences shown in Fig. 6 agree well with similar dependences for alumina powders of a different nature [10]. This clearly points to the electrostatic nature of the stabilization of the suspensions.

A comparison of the regularities in Fig. 6 with the above data of the effect of pH on the brightness of the US image of the suspension (see Fig. 4) shows that there is no direct correlation between the aggregate size in the suspension and its echo-contrast properties. The US response of the suspension decreases both in the pH range corresponding to the minimum aggregation (pH < 6) and the pH range corresponding to the maximum aggregation (pH > 8). The maximum image brightness is observed in the pH range of 6.3–7.4, which corresponds to a nanoparticle aggregate size of 300–600 nm.

Figure 7 shows plots of dependences of the reflected echo-signal brightness on the alumina concentration in the suspension for different biological media. One can see that the intensity of the suspension echo signal decreases significantly upon a decrease in the suspension concentration. Thus, the series of suspensions based on the physiological solution manifests a decrease in brightness from 77.4% at a nanopowder concentration of 4% to 50.5% at a concentration of 0.7%. A similar dependence is observed in solutions with an additive of plasma or blood serum.

It is significant that the average image brightness at any given nanopowder concentration in the suspension is highest for the physiological solution and lowest for the solution containing blood plasma. Figure 8 illustrates this conclusion and shows video images of a silicon tube filled by the nanoparticle suspension based on the physiological solution and solutions containing serum or plasma. At a similar concentration of nanoparticles in suspensions, one can observe an increase in US image inhomogeneity under the addition of blood serum and plasma, accordingly, into the physiological solution.

Figure 9 shows the variation in the weighted average hydrodynamic diameter of Al_2O_3 aggregates when a 5% suspension in the physiological solution is mixed with (*1*) blood serum and (*2*) plasma. The point on the

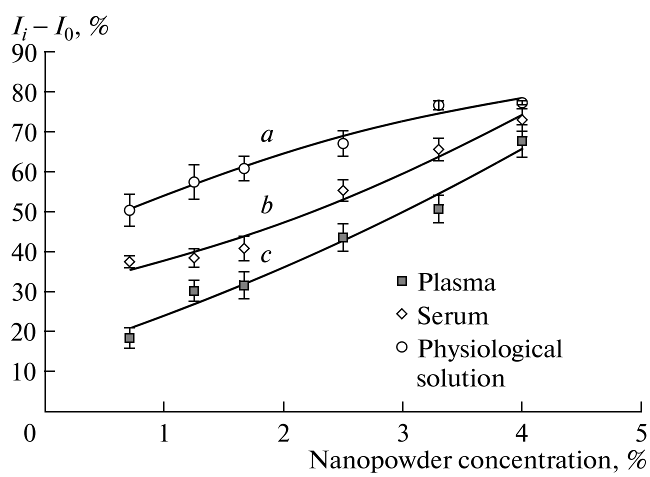

Fig. 7. Echo contrast properties of alumina nanopowder suspensions (*a*) prepared on the basis of the physiological solution, with the addition of (*b*) blood serum and (*c*) plasma. *y* axis: average brightness of the ultrasonic image; *x* axis: nanopowder concentration in the suspension.

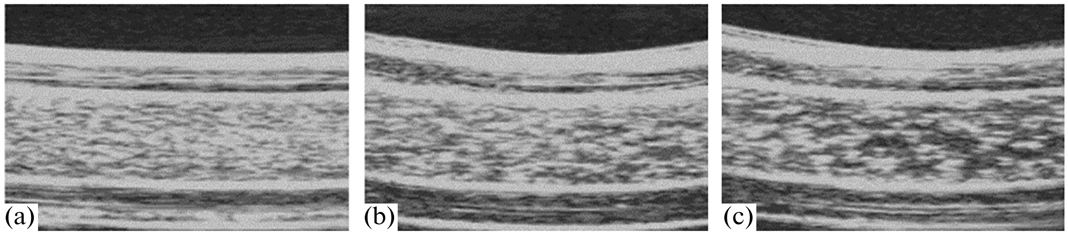

Fig. 8. Ultrasonic images of a vessel filled with a nanoparticle suspension based on (a) the physiological solution, (b) serum, and (c) blood plasma. The concentration of nanoparticles in the suspensions was 2.5%.

right vertical axis corresponds to the weighted average size of aggregates in a suspension in the physiological solution; the points on the left axis correspond to the weighted average hydrodynamic diameter of aggregates in blood serum and plasma free of Al_2O_3 nanoparticles. Though there are no nanoparticles in the system in the latter case, the average aggregate diameter is rather large and is 330 nm for serum and 390 nm for plasma. These sizes correspond to aggregates of the protein structure present in serum and plasma.

As can be seen from the data in Fig. 9, both in the case of blood serum and plasma, the weighted average hydrodynamic diameter of aggregates in suspensions in a mixed medium decreases when compared to the value typical for the physiological solution. The dependence of the weighted average size for the addition of either serum or plasma is described by the same curve.

Unfortunately, the available information is insufficient for the establishment of the unambiguous cause of the observed decrease in the aggregate size. On the face of it, this can be the result of the simple averaging of dimensions of alumina aggregates and protein serum–plasma aggregates. However, scattering on large particles generally screens scattering on small particles in a suspension with particles with different sizes, because the scattering intensity depends on the sixth power of the radius.

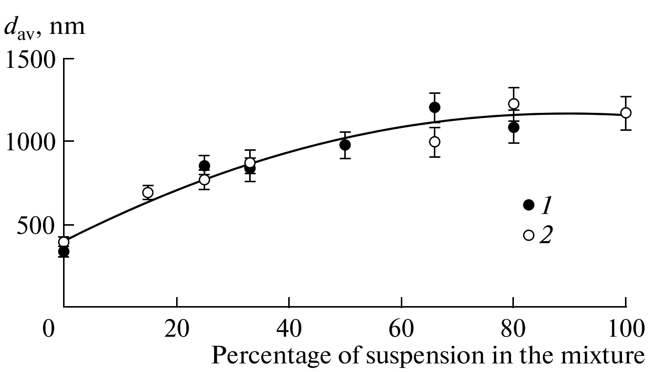

Fig. 9. Dependence of the weighted average particle diameter on the amount of nanopowder suspension added to (*1*) serum and (*2*) blood plasma.

The absolute value of scattering in transparent media (serum and plasma) is much lower than in opalescent alumina suspensions. Therefore, the addition of weakly scattering protein aggregates to a strongly scattering alumina suspension must not result in a decrease in the measured hydrodynamic diameter. Another reason for the decrease in the aggregate size can be the disaggregation of secondary aggregates of alumina nanoparticles due to the formation of an adsorption layer of protein blood serum and plasma components, resulting in the additional steric stabilization of the suspension. More detailed studies are required to elucidate this issue.

DISCUSSION

The acoustic properties of suspensions of alumina nanoparticles in biological media in US fully corresponding in characteristics to the methods of medical US diagnostics of organs and tissues are quantitatively estimated in this work. The considerable enhancement of the echo signal was registered in the 2D (B-mode) US visualization of the basic suspension of alumina nanopowder in the physiological solution.

The video image brightness of suspensions proved to depend nonlinearly on the pH of the solution. Herewith, this dependence had a maximum in the pH range of 6.3–7.4 (see Fig. 4). The pH values are close to the normal acid–alkali balance of blood from 7.25 to 7.45. Therefore, the pH of blood promotes the maximum use of echo-contrast options of alumina nanopowder suspensions.

According to the data on the distribution of hydrodynamic diameters of nanoparticle aggregates as dependent on the pH of the solution (see Fig. 6b), our result agrees well with the earlier established fact of the effect of the aggregation degree of nanoparticles on the echo contrast properties of aqueous alumina suspensions [6]. Accordingly, an increase in the echo-signal intensity under a variation in pH from 2 to 6.3 results from an increase in the aggregation degree of nanoparticles in the suspension. The further increase in the hydrogen indicator values from 7.4 to 11 leads to a decrease in the ultrasonic image brightness. One should point out that the highest brightness of the reflected echo signal corresponds to the initiation of aggregation in the alumina suspension at an increase in the pH value. In this pH range (6.3–7.4), nanoparticle aggregates with drastically different sizes are coexistent. The possibility of regulating nanoparticle aggregation in water based on pH and ionic strength was also shown in other studies [11, 12].

The physical reason for the dependence can be explained on the basis of the known concepts of the character of interaction of ultrasound with colloid suspensions [10]. It is known that the ultrasonic response can be divided into the sum of two contributions, both of which feature an extremum as dependent on the ratio of the (k/d) of acoustic wave wavevector k and particle diameter d in the suspension. The first contribution is related to different processes of ultrasound absorption by particles that result, in their turn, in thermal, viscosity, and other losses. This contribution is predominant at a US frequency below 20 MHz. At frequencies above 100 MHz, the contribution related to the US wave-scattering processes prevails [10].

In US diagnostics, solely US wave absorption is implemented at the frequency of 5 MHz. It is significant that it depends on the k/d ratio featuring a curve with a maximum. This curve, which is usually characteristic for a specific suspension, is constructed in the form of a dependence of the US wave frequency at a constant particle size in the suspension. In our experiment, a different case is implemented: the US frequency remained constant, but the particle size changed as a result of the aggregation processes under pH variation. That is, an increase in aggregate size d under an increase in pH (see Fig. 6b) at constant k results in a gradual decrease in the k/d ratio. Obviously, the range of k/d variation overlaps with the region of the US wave-absorption maximum in a suspension of alumina nanoparticle aggregates. The fact that it is observed in the range of the physiological standard pH is probably due to the specific features of the interaction between aggregates of different sizes and US.

The presence of a biological medium consisting of several thousand organic components (organic acids, sugars, proteins, lipids, etc.) can produce a special effect on the surface charge balance. As can be seen from the results of our study (see Fig. 7), an increase in the content of suspension and blood plasma proteins leads to a decrease in the echo-signal intensity. On the basis of the above, one can assume that this is related to a decrease in the degree of aggregation of nanoparticles in plasma- and serum-containing suspensions. The registration of hydrodynamic diameters of particles in suspensions using the method of dynamic light scattering showed with a high probability that nanopowder suspensions were stabilized in the presence of a biological medium, which resulted in a decrease in the amount of aggregates larger 2 μm and an increase in the amount of small aggregates with diameters not exceeding 500 nm. Herewith, the tendency of nanoparticles for aggregation is lower in plasma when compared to serum and lower than in a suspension based on the physiological solution. The data agree with the results of studying the aggregation of gold nanoparticles in biological liquids [13], where it was shown that the tendency of nanoparticles to aggregate in whole blood and plasma was lower than in aqueous solutions and in a phosphate buffer solution.

Two important cases of the interaction between nanoparticles and biological liquids are described in the literature [14]. Firstly, nanoparticles can be covered by a "crown" of protein molecules and other biomacromolecules and, secondly, they can aggregate, forming clusters of different sizes. Thus, an increase of the hydrodynamic nanoparticle diameter was shown for plasma, which is explained by the formation of a shell of plasma proteins around the nanoparticle [15]. However, we observe a decrease in the cluster aggregation degree alongside an increase in the diameter of individual nanoparticles. Therefore, one can assume that blood proteins bearing, in most cases, a negative surface charge [14] affect the nanoparticle surface charge, suppressing the aggregation of nanoparticles in the suspension.

Thus, the results presented in this study give grounds to consider the suspension of alumina nanopowders a UV-contrasting agent for biological media, in particular, blood. The obtained regularities can serve as a basis for the development of theoretical models of interaction between metal oxide nanoparticles and biological molecules.

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REFERENCES

1. P. A. Dayton and K. W. Ferrara, "Targeted imaging using ultrasound," J. Magn. Res. Imag. **16** (4), 362–377 (2002).
2. S. R. Cherry, "In vivo molecular and genomic imaging: new challenges for imaging physics," Phys. Med. Biol. **49** (3), 13–48 (2004).
3. D. Vancraeynest, X. Havaux, A. Pasquet, et al., "Myocardial injury induced by ultrasound-targeted microbubble destruction: evidence for the contribution of myocardial ischemia," Ultrasound Med. Biol. **35** (4), 672–679 (2008).
4. J. Ophir, A. Gobuty, R. E. McWhirt, and N. F. Maklad, "Ultrasonic backscatter from contrast producing collagen microspheres," Ultrason. Imag. **2** (1), 67–77 (1980).

5. K. J. Parker, T. A. Tuthill, R. M. Lerner, and M. R. Violante, "A particulate contrast agent with potential for ultrasound imaging of liver," Ultrasound Med. Biol. **13** (9), 555–566 (1987).

6. T. F. Shklyar, O. A. Toropova, A. P. Safronov, et al., "Acoustical properties of metal oxides water suspensions," Ross. Nanotekhnol. **5** (3–4), 24–30 (2010).

7. F. A. Blyakhman, et al., RF Patent No. 2444296 C2 IPC**51** A 61B10/00, Byull. Izobret., No. 7 (2012).

8. Yu. A. Kotov, "Electric explosion of wires as a method for preparation of nanopowders," J. Nanopart. Res. **5** (5–6), 539–550 (2003).

9. A. P. Safronov, D. V. Leiman, D. N. Blagodetelev, Yu. A. Kotov, A. V. Bagazeev, and A. M. Murzakaev, "Aggregation of air-dry powders of aluminium oxide nanoparticles under secondary dispergation in water medium," Ross. Nanotekhnol. **5** (11–12), 17–23 (2010).

10. A. S. Dukhin and P. J. Goetz, *Ultrasound for Characterizing Colloids* (Elsevier, 2002).

11. S. W. Bian, I. A. Mudunkotuwa, T. Rupasinghe, and V. H. Grassian, "Aggregation and dissolution of 4 nm ZnO nanoparticles in aqueous environments: influence of pH, ionic strength, size, and adsorption of humic acid," Langmuir **27** (10), 6059–6068 (2011).

12. J. M. Berg, A. Romoser, N. Banerjee, R. Zebda, and C. M. Sayes, "The relationship between pH and zeta potential of 30 nm metal oxide nanoparticle suspensions relevant to *in vitro* toxicological evaluations," Nanotoxicol. **3** (4), 276–283 (2009).

13. A. D. Levin, E. M. Rukin, E. I. Gershman, A. V. Aseichev, L. A. Zakharova, and V. I. Mel'nikova, "The way to characterize of nanoparticles in biological liquids and tissues by means of optical-spectral methods," in *Proc. 2nd Int. Forum on Nanotechnologies* (Moscow, 2009) http://www.rusnanoforum.ru

14. A. E. Nel, L. Mädler, D. Velegol, et al., "Understanding biophysicochemical interactions at the nano-bio interface," Nature Mater. **8** (7), 543–557 (2009).

15. M. A. Dobrovolskaia, A. K. Patri, J. Zheng, J. D. Clogston, N. Ayub, P. Aggarwal, B. W. Neun, J. B. Hall, and S. E. McNeil, "Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles," Nanomed. **5** (2), 106–117 (2009).

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